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MICROFLUIDIC MIXERS FOR THE INVESTIGATION OF PROTEIN FOLDING USING SYNCHROTRON RADIATION CIRCULAR DICHROISM SPECTROSCOPY

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ABSTRACT

The purpose of this study is to design, fabricate and optimize microfluidic mixers to investigate the kinetics of protein secondary structure formation with Synchrotron Radiation Circular Dichroism (SRCD) spectroscopy. The mixers are designed to rapidly initiate protein folding reaction through the dilution of denaturant. The devices are fabricated out of fused silica, so that they are transparent in the UV. We present characterization of mixing in the fabricated devices, as well as the initial SRCD data on proteins inside the mixers.

INTRODUCTION

An improved understanding of how proteins fold into their secondary structure may have a significant impact in the prevention and treatment of various diseases. Use of microfluidic mixers with a variety of spectroscopic techniques such as single molecule [1], and ensemble FRET [2], SAX [3,4], FTIR [5] has improved the time resolution and greatly reduced sample consumption over more conventional stopped flow mixing methods. Circular dichroism (CD) is a spectroscopic technique commonly used for studies of protein folding that so far has not been used with microfluidic devices. In CD spectroscopy, linearly polarized light is incident on an optically active protein. Linearly polarized light consists of both left and right circularly polarized light of equal magnitude and phase. An optically active material preferentially absorbs one of these circularly polarized components of light. The measured CD signal is this difference in

function of wavelength. Protein structures such as alpha helices and beta sheets can be distinguished in the CD signal. SRCD allows us to use wavelengths below 220 nm where differences between the CD spectra of random coil and the various secondary structure types are most pronounced. Microfluidic mixing allows a fast initiation of the protein folding reaction. By combining microfluidic initiation of the folding reaction with observations using SRCD, we will be able to measure structure formation during the early events of protein folding (sub ms). Our research will clarify an intense debate in the protein folding community as to when, in the process of folding, the secondary structure content forms.

EXPERIMENTAL DETAILS

We designed, fabricated and characterized mixing in the microfluidic device prototype. A photograph of a device prototype is presented in Fig. 1. Since at high concentrations, guanidinium hydrochloride denaturant (GuHCl) prevents proteins from folding, one of the most common ways to initiate folding is through rapid dilution of denaturant with buffer. In our device, protein solution with high denaturant concentration is injected into one channel and buffer solution is injected into another channel as shown in Fig. 2. The solutions meet at a serpentine-shaped region depicted in Fig. 3. The serpentine-shaped region performs mixing in the laminar flow regime by virtue of diffusion and chaotic advection [6]. Once the solutions are mixed and the denaturant is diluted, the folding reaction is initiated. The spectroscopic measurements are performed in the "observation channel" downstream.

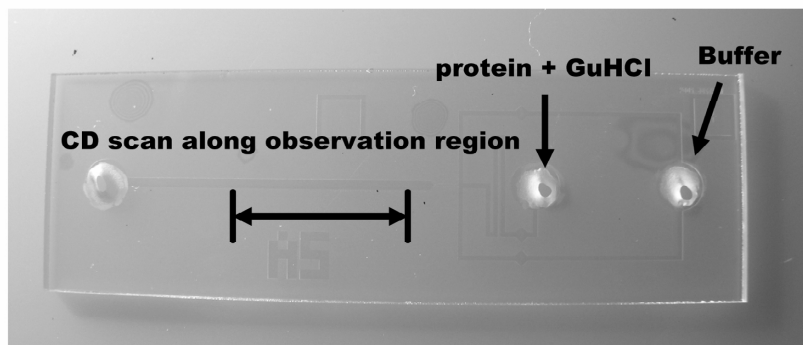


Figure 1: Photograph of fabricated mixer with sandblasted inlet / exit holes.

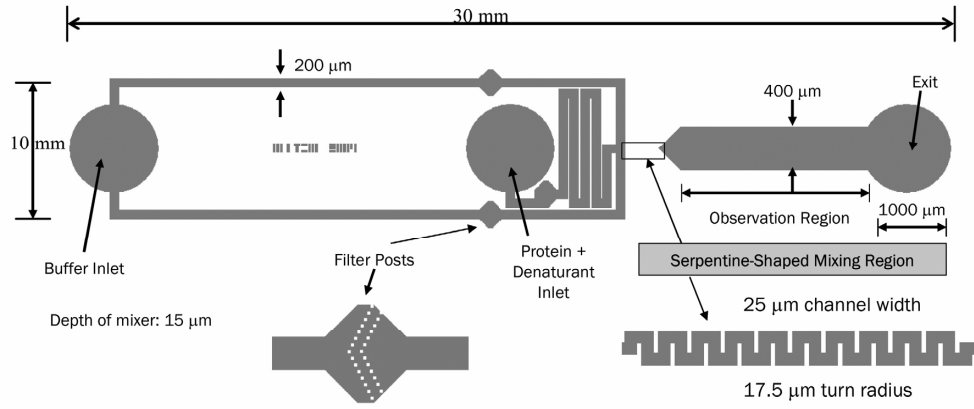


Figure 2: Schematic of the design of SRCD Mixer.

To allow for transparency in the UV range where CD measurements are performed, the mixers are fabricated out of fused silica (Corning 7980, 0F grade). The channels are etched to a depth of 14.5 μm using deep reactive ion etching with nearly vertical sidewalls. Etching is performed with an STS Advanced Oxide Etcher with a selectivity of approximately 17:1 using an undoped polysilicon mask. The inlet holes are fabricated by micro-sand blasting. Sealing of microfluidic mixers is accomplished by direct fusion wafer bonding to another fused silica substrate. Both the etched and unetched wafers were first thoroughly cleaned using piranha solution (sulfuric acid + hydrogen peroxide) and reverse RCA cleaning procedure. Following the piranha etch, substrates were cleaned for 10 minutes in 5:1:1 $\text{H}_2\text{O}:\text{HCl}:\text{H}_2\text{O}_2$ solution at a temperature of 75 $^\circ\text{C}$. The final step is to clean the substrates for 20 minutes in 5:1:1 $\text{H}_2\text{O}:\text{NH}_4\text{OH}:\text{H}_2\text{O}_2$ solution at a temperature of 72 $^\circ\text{C}$. After drying, the substrates were first pre-bonded and then fused at 1100C.

RESULTS

Mixing is observed at various flow rates by measuring the fluorescence intensity of fluorescein dye mixed with buffer. We mounted the mixer chips on a plastic holder that allows us easy connection to a syringe pump (Harvard Apparatus Infusion Syringe Pump 22, Model 55-2222). The fluorescein dye and the buffer were mixed in the ratio 1:1. To quantify the mixing process, we defined a mixing metric, M , based on the standard deviation of the fluorescence intensity as

$$M = 1 - 2\sqrt{\frac{\sum_{i=1}^N (I_{\text{norm},i} - I_{\text{avg}})^2}{N}}$$

Value of mixing metric $M=0$ corresponds to a completely unmixed state, while $M=1$ corresponds to a completely mixed state. Intensity scans were taken at various flow rates to measure M as shown in Fig. 4A-4D. Our experiments demonstrate that, as expected, at progressively higher flow rates, Dean and Corner vortices interact to stretch and fold streamlines, thus enhancing mixing (Figure 4E).

Figures of merit for the performance of the mixers for protein folding measurements are: 1) mixing metric M ; 2) mixing time, 3) time resolution & 4) sample consumption. The mixing time corresponds to the time it takes for the solution to traverse the serpentine mixing region. The time resolution is determined as the time the protein solution spends in the beam. That time is

calculated as the ratio of the width of the beam spot ($\sim 50\mu\text{m}$) and the average flow velocity in the observation region. A plot of the mixing metric M , mixing time and time resolution as a function of the total flow rate is shown in Fig. 5. With the current device design, the fastest mixing time obtained was 50 μs at a flow rate of 400 $\mu\text{l}/\text{min}$. This indicates that we will be able to observe folding events that happen at time scales $>50\mu\text{s}$.

Initial evaluation of the microfabricated mixers at the BESSY II synchrotron beam (Berlin, Germany) has determined that the mixers are suitable for measurements of protein folding kinetics. The SRCD data presented in Fig. 6 is of the filtered lysozyme protein solution inside the microfluidic device. The data shows an acceptable signal-to-noise ratio, which confirms that the developed etch process results in a smooth enough fused silica surface that does not interfere with the polarization of the CD signal.

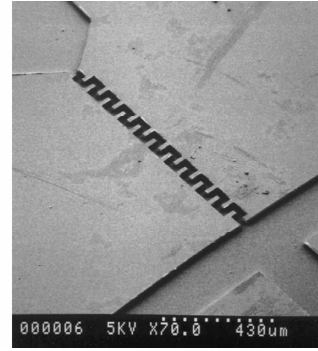


Figure 3: Scanning electron micrograph of the mixing region.

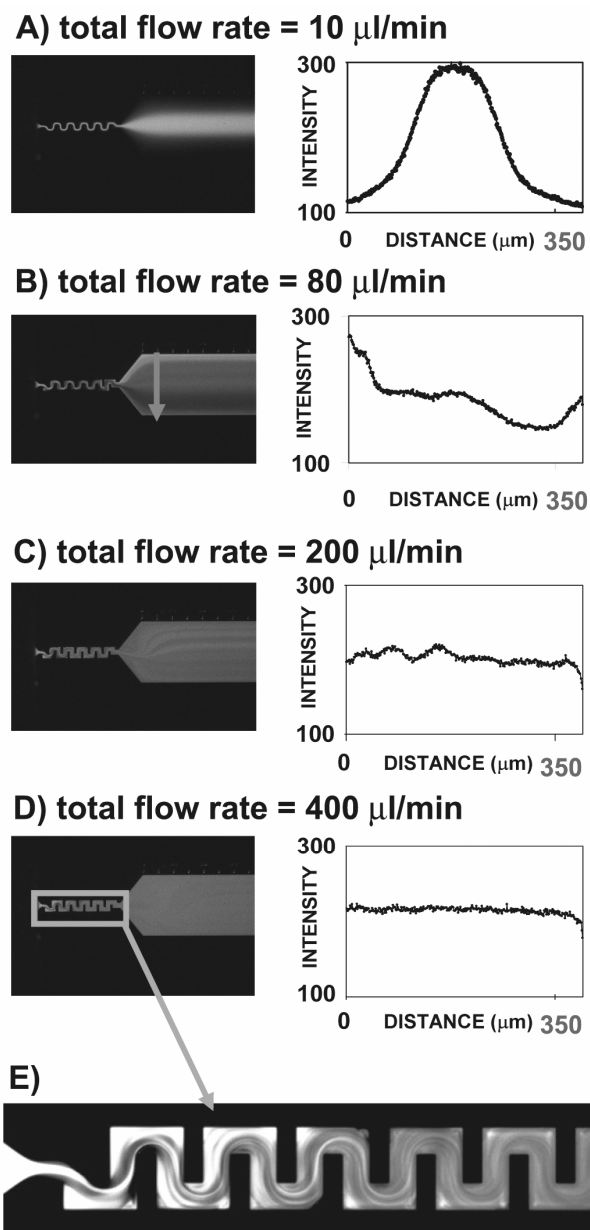


Figure 4: A-D: Fluorescence images of mixing as a function of flow rate. Intensity scans are performed perpendicular to the direction of flow, across the entire width of the observation channel, as indicated by the gray arrow in Fig. 4B. E) Zoom of the mixing region.

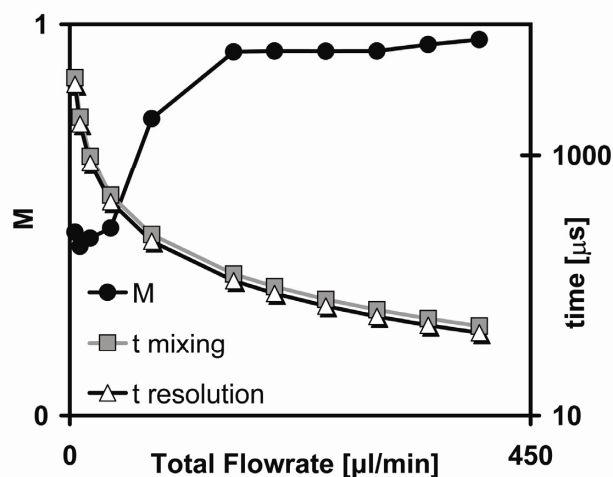


Figure 5: Plot of the mixing metric, mixing time and time resolution as a function of total flow rate.

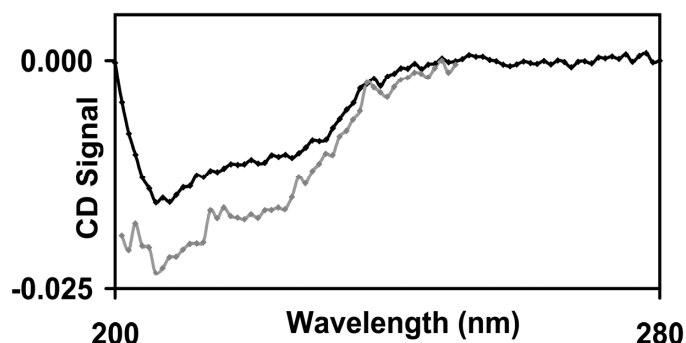


Figure 6: SRCD data of filtered lysozyme protein solution inside microfluidic device. Black curve shows the reference data of lysozyme in water while the gray curve shows the data taken with the lysozyme and 0.6M GuHCl in the mixer. The measurements in the presence of 0.6M GuHCl are limited to about 199 nm because of GuHCl absorption.

CONCLUSIONS AND FUTURE DIRECTIONS

We described the design, fabrication and characterization of the microfluidic devices for measurement of protein folding kinetics using SRCD. We presented the data demonstrating the feasibility of the kinetic experiments with our devices.

Further SRCD measurements will be performed to analyze the kinetics of formation of the protein secondary structure while performing mixing of the protein + GuHCl solution with buffer. The current microfluidic mixers will be optimized in order to achieve low mixing times together with lower sample consumption. Introducing a microfluidic mixer for synchrotron-based spectroscopy opens up additional avenues for research in the biological sciences.

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